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Outcome of Azacitidine Therapy in Acute Myeloid Leukemia is not Improved by Concurrent Vorinostat Therapy but is Predicted by a Diagnostic Molecular Signature

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Statement of translational relevance:

The clinical benefit of azacitidine (AZA) monotherapy in acute myeloid leukemia (AML) is blunted by low response rates and the inevitability of disease progression. Combination therapy with histone deacetylase (HDAC) inhibitors has been proposed to improve outcome but has not been prospectively studied in AML. The demonstration in this randomized study that co-administration of AZA with the HDAC inhibitor vorinostat does not improve outcome in newly diagnosed or relapsed AML confirms the importance of identifying new therapeutic partners for AZA. In this context the observation that mutations in the cell cycle checkpoint activator CDKN2A correlate with adverse clinical outcome represents the first clinical validation of *in vitro* data implicating induction of cell cycle arrest as a mechanism of AZA's clinical activity informing the design of novel drug combinations. Furthermore, persistence of stem/progenitor populations throughout therapy identifies their role as a biomarker of response to AZA based regimens.

Abstract

Purpose: Azacitidine (AZA) is a novel therapeutic option in older patients with acute myeloid leukemia (AML) but its rational utilization is compromised by the fact that neither the determinants of clinical response nor its mechanism of action are defined. Co-administration of histone deacetylase inhibitors, such as vorinostat (VOR), is reported to improve the clinical activity of AZA but this has not been prospectively studied in AML. **Experimental Design:** We compared outcomes in 259 adults with AML (n=217) and MDS (n=42) randomized to receive either AZA monotherapy (75 mg/m^2 × seven days every 28 days) or AZA combined with VOR 300 mg bd on days 3-9 po. Next generation sequencing was performed in 250 patients on 41 genes commonly mutated in AML. Serial immunophenotyping of progenitor cells was performed in 47 patients. **Results:** Co-administration of VOR did not increase the overall response rate (*P*=0.84) or overall survival (OS) (*P*=0.32). Specifically, no benefit was identified in either *de novo* or relapsed AML. Mutations in the genes *CDKN2A* (*P*=0.0001), *IDH1* (*P*=0.004) and *TP53* (*P*=0.003) was associated with reduced OS. Lymphoid multi-potential progenitor populations were greatly expanded at diagnosis and although reduced in size in responding patients remained detectable throughout treatment. **Conclusion:** This study demonstrates no benefit of concurrent administration of VOR with AZA but identifies a mutational signature predictive of outcome after AZA based therapy. The correlation between heterozygous loss of function *CDKN2A* mutations and decreased OS implicates induction of cell cycle arrest as a mechanism by which AZA exerts its clinical activity.

Introduction

The DNA methyltransferase (DNMT) inhibitors azacitidine (AZA) and decitabine (DEC) represent important advances in the management of patients with acute myeloid leukemia (AML) and high-risk myelodysplasia (MDS) ineligible for intensive chemotherapy (1). Recent randomized trials have demonstrated that AZA improves outcome in older adults with AML and high risk MDS (2, 3). More recently AZA has been to shown to possess significant clinical activity in relapsed and refractory AML (4-6). However, the clinical utility of AZA in both newly diagnosed and advanced disease is limited by relatively low rates of complete remission (CR) and the fact all patients relapse despite continuing therapy. There is consequently an urgent need to identify novel therapies with the potential to improve the outcome after AZA monotherapy. Co-administration of AZA with histone deacetylase (HDAC) inhibitors augments killing of leukemic cell lines *in vitro* (7) and single-arm trials have described increased clinical activity of AZA in combination with a number of HDAC inhibitors including sodium valproate and vorinostat (VOR) (4, 8, 9). Although recent randomized trials have reported no benefit of co-administration combined AZA and HDAC inhibitor therapy in high-risk MDS there have been no randomized trials in AML (10-12).

The development of pharmacological strategies to improve the outcome of AZA based therapy in AML has been hampered by our limited understanding of its mechanism of action. Whilst *in vitro* and animal studies demonstrate that induction of cell cycle arrest and up-regulation of cell cycle genes correlates with AZA's anti-leukemic activity the mechanism by which it exerts a clinical anti-tumor effect remains a matter of conjecture (7). Furthermore, although disease progression appears inevitable in patients treated with AZA little is understood of the mechanism

of disease resistance (13). Recent immunophenotypic characterization of the stem/progenitor cell compartments containing leukemic stem cells (LSCs) in AML have demonstrated their persistence in a proportion of patients consistent with the hypothesis that this cellular population represents a reservoir of resistant disease although this proposition has not been prospectively examined in AZA treated patients (4, 14).

We therefore examined whether co-administration of the HDAC inhibitor VOR increases response rates and OS in patients with AML and high risk MDS and correlated these clinical endpoints with both diagnostic genotypes and serial stem/progenitor quantitation.

Subjects and Methods

Trial design: RAvVA was a multi-center, open label, prospective randomized phase II trial designed to assess the activity and safety of AZA monotherapy compared to combined AZA and VOR therapy in AML and high-risk MDS patients (ISRCTN68224706, EudraCT 2011-005207-32) delivered by the Bloodwise Trials Acceleration Program (TAP).

Patients: Patients with newly diagnosed, relapsed or refractory AML as defined by the World Health Organization (WHO) Classification or high-risk MDS (IPSS INT-2 or high-risk) according to the International Prognostic Scoring System (IPSS) deemed ineligible for IC on the grounds of age or co-morbidities were eligible for inclusion in this trial (Table 1) (15). A high presentation white count was not an exclusion to trial entry and patients were permitted to receive hydroxycarbamide after AZA administration for the first cycle of therapy. All patients required adequate renal and hepatic function and an Eastern Cooperative Oncology Group (ECOG) performance status ≤2 as a condition of trial entry. Patients with acute promyelocytic leukemia, a

prior allogeneic stem cell transplant or prior treatment with AZA or other DNMT inhibitors were ineligible.

Treatment regimens: Patients were randomized on a 1:1 basis using a minimization algorithm with three variables: disease category (AML vs MDS), stage of disease (newly diagnosed vs relapsed / refractory) and age (<70 vs 70+). Patients in the control arm received AZA (75mg/m^2) by subcutaneous (SC) injection on a five-two-two schedule, commencing on day one of 28-day cycles for up to six cycles. In the combination arm patients received the same schedule of AZA in conjunction with additional VOR (300mg bd) orally (po) for seven consecutive days commencing on day three of each cycle. All study participants achieving a CR, CR with incomplete blood count recovery (CRi), marrow CR (mCR) or partial response (PR) within the first six cycles of treatment, were permitted to continue study treatment until loss of response. Non-responding patients discontinued trial therapy. Bone marrow samples for morphology and immunophenotypic assessment were collected after cycles three and six and three months thereafter. Compliance to treatment was defined as the number of patients who received treatment as planned according to the trial protocol.

Efficacy endpoints: Two primary endpoints were defined: overall response rate (ORR) and overall survival (OS). ORR was defined as acquisition of CR, CRi, mCR or PR within six cycles of treatment utilizing modified Cheson or IWG criteria (16, 17). For each patient the response after the third and sixth cycle of trial treatment was reviewed and the better of the two was considered the ‘best response’ and used here. OS was defined as the time from date of randomization to the date of death from any cause. Secondary outcome measures included CR/ CRi/ mCR rate within six cycles of trial therapy, duration of response defined as time from response to relapse, dose intensity defined as the total dose prescribed to each patient as a

proportion of the protocol dose and NCI CTCAE v4 defined grade ≥ three adverse event or SAE. Induction death was defined as death prior to the first response assessment.

Next Generation Sequencing (NGS) and Bioinformatic analysis: Bone marrow aspirates were collected at diagnosis on consenting patients. Mutational analysis using NGS was performed on 250 diagnostic bone marrow samples. Genomic DNA was subjected to multiple x PCR on the Fluidigm Access Array. The panel consists of 904 amplicons across 41 genes frequently mutated in AML and myeloid malignancies, covering areas with high frequency of AML gene mutations (hotspots), or whole exons if no hotspots were previously reported in COSMIC (Supplemental Tables 1 and 2). Sequencing was performed on an Illumina MiSeq with 300 bp paired-end reads, yielding an average read depth of 912 reads per amplicon. Fluorescent capillary electrophoresis was performed in addition to NGS for the detection of FLT3 internal tandem duplications (ITDs), since the rate of detection of ITDs is ~60% using NGS alone (18). Details of Next Generation Sequencing methodology is provided within Supplementary Information (Supplementary Tables 1, 2 and 3).

Bioinformatic analysis

Sequencing quality was assessed using FASTQC (Samtools) and aligned using a Burroughs-Wheeler Aligner algorithm in Stampy. A Phred score of 30 was set as a minimum quality threshold for variant calling. We used 2 variant callers: VARSCAN and Pindel, using the following parameters: minimum coverage 100 reads; minimum variant frequency 0.05; minimum read depth of variant 5; P value 0.05. As germline DNA was not available, we implemented criteria to optimize calling of disease-

associated mutations and to exclude likely germline single-nucleotide polymorphisms or technical artefacts.

Inclusion criteria for variant calling and filtering were as follows: (i) mutations in protein coding regions or conserved splice sites; and (ii) previous documentation as a somatic mutation in hematopoietic samples in COSMIC with a minimum variant allele frequency (VAF) of 0.05; or (iii) novel truncating variants (nonsense, deleterious missense/indels, variants affecting splicing) with a minimum VAF of 0.05; or (iv) novel single nucleotide variants with a minimum variant frequency of 0.1, if they cluster within 3 codons of a previously documented somatic variant reported in COSMIC or in the large AML dataset of Papaemmanuil NEJM 2016 (REF) ; v) SNVs with a VAF > 0.1 which did not meet the exclusion criteria below.

Exclusion criteria for variants were the following: (i) variants predicted to result in a silent amino acid change; (ii) known polymorphisms present in human variation databases at a population frequency of > 0.0014 (0.14% reflecting the population incidence of myeloid disease); (iii) 1-bp indels present adjacent to regions of 4 homopolymer bases at < 0.2 variant frequency; (iv) variants that occur in > 3 samples of our cohort at a VAF of 0.05 to 0.1 that are not previously documented in COSMIC, which likely constitute PCR or sequencing artefacts in genomic regions prone to error. All putative variants were further validated by visualization using the Integrated Genome Viewer.

Stem/progenitor immunophenotypic quantitation: Bone marrow (BM) aspirate samples were collected for sequential quantification of leukemic stem/progenitor populations pre-treatment, during treatment and at relapse. Mononuclear cells were isolated by Ficoll density gradient and viably frozen using 10% DMSO and stored in liquid nitrogen. Frozen mononuclear cells (MNCs) from BM samples were thawed on

the day of analysis, washed with Iscove's Modified Dulbecco's Medium (IMDM, Thermo Fisher Scientific, UK) supplemented with 10% fetal bovine serum (Sigma, UK) and 1mg/ml bovine pancreatic DNAse I (Sigma, UK). Cells were stained for FACS analysis as detailed below. FACS analysis was carried out on either BD LSR Fortessa or a BD FACSAria Fusion (Becton Dickinson, Oxford UK).

Antibodies used in the lineage (Lin) depletion cocktail were: anti-CD2, anti-CD3, anti-CD4, anti-CD8a, anti-CD10, anti-CD19, anti-CD20 and anti-CD235a. Antibodies used to analyze different subpopulations were: anti-CD34, anti-CD38, anti-CD90, anti-CD45RA, anti-CD123, anti-CD117, and 7AAD were used as a live/dead stain. Details of each antibody/streptavidin are listed in Supplemental Table 4. We did not deplete CD11b, CD14, CD7, CD56 expressing cells as these markers may be expressed by CD34+ and CD34-CD117+ LSC populations. We assessed LSC populations pre-treatment, during treatment and in a subset of patients, at relapse. Patients were selected for longitudinal LSC assessment based on availability of appropriate viably-banked samples and documented clinical outcome. The size of the stem/progenitor subpopulations of each sample were determined as a percentage of live Lin^- MNCs, and expressed as a fold change of the upper limit values of normal control bone marrow (Supplemental Table 5). An example of the gating strategy is demonstrated in Supplemental Figure 1.

Statistical analysis: The sample size was calculated on conventional phase III criteria but with a relaxed alpha and was originally powered to recruit 160 patients (ORR: P_0= 15%, detectable difference=15%, 2-sided α and β =0.15, 0.2 respectively, OS control rate at one year=15%, detectable difference of 15%, 2-sided α and β=0.1, 0.2 respectively). At two years based on the advice of the DMC the sample size was updated to 260 (ORR: P_0=0.2, detectable difference=0.15, 2-sided α=0.1, β=0.15,

OS: P_0=0.15, detectable difference=0.15, 2-sided α=0.1, β<10%). Given the increased sample size the final trial had sufficient power to investigate a trend of activity for both ORR and OS in a predetermined subgroup analysis of the newly diagnosed and advanced disease groups (detectable difference in ORR and OS=20% and 20% respectively, 2-sided α=0.2, β=0.2).

Standard statistical methods were used for all analysis in the trial: Fisher exact or chi-squared tests for categorical endpoints (e.g. response), Kaplan Meier curves and log rank tests for time-to-event endpoints (e.g. survival), cumulative incidence curves and Cox models for time to event endpoints with competing risks (e.g. time to first response). In order to investigate clinical factors predicting outcome after AZA based therapy we performed logistic and Cox multivariable analysis on all trial patients for ORR and OS including treatment arm as a co-variable. Sub group analysis is presented using forest plots with test for interaction displayed. *P* values of 0.1 were considered significant for both primary outcomes and a *P* value of 0.05 was considered significant throughout the rest of the analysis. No multiple testing adjustments has been carried out as the analysis conducted was exploratory and hypothesis generating. **Results**

Baseline characteristics of patients: Between November 2012 and September 2015 260 patients were recruited from 19 UK centers as outlined in the consort diagram (Figure 1). One patient was randomized for a second time in error and removed from analysis. Baseline patient characteristics are listed in Table 1. Two hundred and seventeen patients had a diagnosis of AML at time of randomization and 42 patients with MDS. Of the 217 patients with AML 111 were newly diagnosed, 73 had relapsed disease and 33 were refractory to at least one prior line of therapy.

Treatment administration and toxicities: Patients received a median of six cycles (IQR: 2, 8) of treatment in both arms of the trial. Average compliance to AZA across all cycles of treatment was 73% in the AZA arm and 71% in the combination arm. There was no difference in dose intensity across treatment arms with a median intensity of 100% of the dose delivered in the first six cycles of treatment. 106 patients in the AZA arm experienced one or more toxicity compared to 110 patients in the combination arm and there was no difference between treatment arms (*P*=0.87). Adverse events (Grade 3 and 4) experienced by 5% or more of patients are listed in Supplemental Table 6.

Response and survival: There was no difference in either ORR (41% versus 42%) (OR=1.05 (95% CI: 0.64, 1.72), *P*=0.84) or CR/CRi/mCR rate (22% and 26%) (OR=0.82 (95% CI: 0.46, 1.45), *P*=0.49) between the control and combination therapy arms. Time to first response and duration of response at one year was similar in the AZA and combination arm (6.2 vs 5.7 months and 67% vs 58% respectively) (Supplemental Figure 2). In pre-determined subgroup analysis patients with relapsed/refractory disease demonstrated an increased CR in the AZA/VOR arm (*P*=0.02), although this did not translate to an improvement in OS.

No difference was observed in OS between patients treated with AZA monotherapy (median OS: 9.6 months (95% CI: 7.9, 12.7)) and patients in the AZA/VOR arm (median OS: 11.0 months (95% CI: 8.5, 12.0)) (HR=1.15 (95% CI: 0.87, 1.51), *P*=0.32). Specifically, there was no difference in OS between treatment arms in patients with newly diagnosed or relapsed/refractory AML (Figure 2)(Supplemental Figure 3).

Clinical and molecular factors predicting outcome after AZA based therapy: We next wished to identify clinical factors predictive of response to AZA based

therapy in the trial cohort. Multivariable logistic regression demonstrated higher ORR rates in newly diagnosed disease vs refractory/relapsed disease (*P*=0.038). Neither diagnosis (AML vs MDS, *P*=0.22) nor presentation karyotype (favorable vs intermediate vs poor, *P*=0.76) predicted ORR in the same model. Cox regression analysis demonstrated increased OS in patients with MDS as opposed to AML (*P*=0.012), a low ECOG score (*P*=0.09) and a presentation WBC $<10\times10^9$/l (*P*=0.019). Presentation karyotype did not correlate with OS.

The impact of diagnostic mutational status on clinical response and OS was then studied using the results of NGS performed on 250 patients at trial entry (Figure 3A). The mean mutation number per patient was 3.4 (Figure 3B). Mutations in *RUNX1* were most frequent (73 patients, 29%). Mutations in *DNMT3A* (59 patients 23%), *IDH2* (57 patients, 23%) and *TET2* (56 patients, 22%) were also common (Figure 3A). The observed mutational frequency was broadly consistent with that previously reported in older, but not younger, AML and MDS patients(19-21) (Figure 3C). In univariate analysis there was a lower complete response (CR, CRi, mCR) rate in patients with an *IDH2* mutation (*P*=0.029) and *STAG2* mutation (*P*=0.002) but an increased CR rate in patients with an *NPM1* mutation (*P*=0.038) (Table 2). When considered in a multivariable analysis adjusted for all clinical variables, the presence of *STAG2* and *IDH2* mutations was not shown to have a significant association with acquisition of CR (Table 2). However, *NPM1* mutation remained of prognostic significance (*P*=0.012).

Mutations in *CDKN2A* (*P*=0.0001), *IDH1* (*P*=0.004), *TP53* (*P*=0.003), *NPM1* (*P*=0.037) and *FLT3-ITD* (*P*=0.04) were associated with reduced OS in univariate analysis. In multivariate analysis adjusted for all clinical variables, mutations in *CDKN2A*, *IDH1* and *TP53* were associated with decreased OS (Table 2). No

mutations were associated with improved OS. Mutations in *ASXL1* (*P*=0.035) and *ETV6* (*P*=0.033) were associated with a reduced duration of response. No mutations were associated with improved duration of response.

Amongst other frequently co-occurring mutations, we observed significant co-occurrence of *NPM1* mutations with *DNMT3A*, *FLT3-ITD*, *FLT3-other* and *IDH1* as well as *DNMT3A* with *FLT3-other*, *IDH1* and *IDH2* (*P*<0.05 for all comparisons). Patients with mutations in both *DNMT3A* and *IDH1* had reduced OS (median OS 9.8 months, 95% CI: 1.5-11.6 months) compared to patients without both mutations (median OS 10.7, 95%CI: 8.9-12). Patients with both *NPM1* and *IDH1* mutations had reduced OS (median OS 3.8 months, 95%CI: 1.6-NE) compared to patients without both mutations (median OS 10.7, 95%CI: 9.0-11.8). No significantly co-occurring mutations were found to be predictors of acquisition of CR (Figure 3D).

Impact of AZA based therapy on the LSC population: An expanded $CD34^+$ progenitor population was observed in 42/45 studied patients at diagnosis, while a $CD34^-$ expanded precursor population was observed in 3/45 (Figure 4A). The majority of expanded populations were lymphoid-primed multi-potential progenitors (LMPP: Lin-CD34+CD38-CD90-CD45RA+), which have been previously characterized as an LSC population with functional leukemia-propagating activity in serial xeno-transplant assays(14), and as a novel biomarker of AML disease response and relapse(4). Quantitatively, the immunophenotypic LMPP population is usually very small in normal bone marrow (<2 in 10^5 cells – Vyas et al data under review). Therefore, expansion of the LMPP population can be a sensitive measure of residual disease at CR in AML patients. For these reasons, we focused on quantitation LMPP by immunophenotyping to measure the impact of therapy on putative LSC populations at best response and relapse.

In seven patients with resistant disease, there was no reduction in LMPP numbers measured as a fold change (Figure 4B). Of interest, there was no significant reduction of LMPP numbers in eight patients achieving a PR, where the average bone marrow blast percentage was reduced by 50%. In contrast, in 22 CR/Cri/mCR patients there was a significant reduction in LMPP numbers with AZA-based therapy. However, even here LMPP numbers failed to normalize in 16/22 (Figure 4B). In seven patients with expanded LMPP numbers, who achieved a CR, sequential monitoring demonstrated expansion progenitor populations prior to disease relapse (Figure 4C).

Discussion

Co-administration of the HDAC inhibitor VOR did not improve response or survival in patients with AML or MDS treated with AZA. This observation is consistent with previous randomized studies in high risk MDS but is the first demonstration that HDAC inhibitors have no impact on clinical outcomes in patients with newly diagnosed or relapsed AML treated with AZA (10-12). Why might our study have failed to replicate earlier single arm studies of strikingly increased clinical activity of combined AZA and HDAC inhibitor treatment (8, 9, 22)? Clinical and molecular characterization demonstrates comparability between study arms and confirms that the trial population was broadly representative of older patients with high-risk AML and MDS. Alternatively, the clinical activity of the experimental study arm might have been blunted because VOR associated drug toxicity resulted in under-dosing of AZA. Detailed pharmacovigilance excluded this possibility and indeed AZA dose intensity was similar in both treatment arms. Consideration should however be given to the possibility that co-administration of HDAC inhibitors might inhibit cellular uptake of aza-nucleosides and exploration of alternative dosing schedules may be worth exploring.

The search for novel drug partners with the potential to improve the clinical activity of AZA has been hampered by the fact that its mechanism of clinical activity remains unknown. Cell line and animal data have identified up-regulation of epigenetically silenced genes and consequent restoration of cell cycle checkpoints as an important potential mechanism of action and indeed previous *in vitro* studies have correlated the ant-tumor activity of both AZA and DEC with their ability to effect changes in cell cycle gene expression and induce G2 phase arrest (7, 23, 24). Consequently, the observation that heterozygous predicted loss of function mutations in *CDKN2A*, a

cell cycle checkpoint activator, are correlated with decreased survival in AZA treated patients is supportive of the hypothesis that induction of cell cycle arrest is a potentially important mechanism of action of this agent. In our study the *CDKN2A* mutations were nonsense in two patients and in the other seven were either non-synonymous SNVs that had previously been reported (six patients) or within two codons of a previously reported mutation (one patient). *CDKN2A* encodes P14, P16 and ARF. P14 and P16 inhibit the cyclin dependent kinase CDK4 which regulates the G1 cell cycle checkpoint. ARF sequesters the E3 ubiquitin-protein ligase MDM2, a protein responsible for the degradation of p53. Thus, if loss of CDKN2A abrogates the clinical activity of AZA it raises the possibility that AZA induces G1 cell cycle arrest and requires at least some p53 function for its anti-leukemic activity. We acknowledge that the findings of this study are based on a small sample size and that it is important to replicate this clinical association of CDKN2A mutations with poor clinical response to AZA in larger studies. If confirmed, our data highlight further study of P14, P16 and ARF function as a potentially fruitful line of investigation in understanding and potentially improving the outcomes of AZA based therapy.

The identification of both clinical and molecular predictors of outcome with AZA therapy is important if this agent is to be optimally deployed. Improved survival noted was observed in patients with newly diagnosed disease, a low presentation white count and ECOG score. Importantly, and in contrast to patients treated with myelosupressive chemotherapy, we observed no impact on survival of an adverse risk karyotype after AZA based therapy (25). Our data also demonstrate that NGS improves risk stratification since mutations in *CDKN2A*, *IDH1* and *TP53* were independently associated with decreased survival in AZA-treated patients. We did not identify any impact of mutations in *TET2* or *DNMT3A* on outcome, in contrast to

previous smaller retrospective studies (26-29). Although *TP53* mutations have previously been shown to be associated with decreased survival in patients treated with intensive chemotherapy (30) it has recently been reported that the presence of a *TP53* mutation was associated with a higher response rate in patients treated with DEC (21). In contrast our data demonstrating no impact of TP53 on response rate to AZA but decreased OS in mutated patients implies that these two DNMT inhibitors may have distinct mechanisms of action (16, 20, 33).

The development of strategies to overcome the inevitability of disease relapse in AML patients treated with AZA is essential if outcomes are to improve. It is postulated that disease recurrence in AML patients treated with either myelosuppressive chemotherapy or DNMT inhibitors occurs as a result of expansion of chemo-resistant LSC. However, correlative data in large cohorts of patients treated with either modality of therapy has been lacking. Thus, the demonstration in this study of LSC persistence in AZA treated patients who achieve a CR is consistent with the hypothesis that this recently identified cellular population may serve as a reservoir of resistant disease in AZA treated patients. These data contrast with observations in patients treated with conventional chemotherapy where durable clearance of LSC appears to correlate with long-term remission and highlight the potential importance of quantitation of this cellular population as a biomarker of response in future studies of novel AZA based combinations (4, 31, 32).

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Authorship

C.C. designed the study, recruited patients and wrote the paper; A.E.H. analyzed patient data, constructed figures and tables and wrote the paper; L.S.Q. supervised genetic and stem/progenitor studies, designed figures, read and corrected the manuscript; P.F. recruited patients and collected patient samples; E.G assisted with study design, data interpretation and manuscript editing; C.R analyzed patient data, constructed figures and tables and wrote the paper; M.M performed and analyzed the genetic studies, constructed figures and tables and wrote the paper, NGM performed and analyzed the stem/progenitor immunophenotyping and drew the figures; A.K. analyzed the genetic studies, constructed figures and tables and wrote the paper, A.H directed the *FLT3 ITD* fragment validation studies, M.R. assisted in study design and recruited patients; S.N. recruited patients and collected patient samples; L.D. collected and analyzed patient data; K.W. designed the study and assisted in data analysis and interpretation; J.D.C., M.D., M.F.M., R.J.K and S.P.P recruited patients and collected patient data; S.S. assisted with study design, data interpretation and manuscript editing; P.V. designed the study, recruited patients and led genetic and stem/progenitor studies.

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Tables

Table 1. Demographics of study population

	Whole population (n=259)		*Azacitidine alone (n=129)*		*Azacitidine + Vorinostat (n=130)*	
	No.	%	No.	%	No.	%
Age, years						
< 70 years old	96	37	48	37	48	37
≥70 years old	163	63	81	63	82	63
Gender						
Male	156	60	75	58	81	62
Female	103	40	54	42	49	38
AML disease stage						
Newly Diagnosed	111	43	57	44	54	42
Relapsed	73	28	34	26	39	30
Refractory	33	13	17	13	16	12
MDS disease stage						
Newly Diagnosed	36	14	16	12	20	15
Relapsed	5	2	4	3	1	1
Refractory	1	0	1	1	0	0
ECOG performance status						
0	84	32	52	40	32	25
1	133	51	63	49	70	54
2	26	10	9	7	17	13
Missing	16	6	5	4	11	8
Cytogenetic group						
Favorable risk	13	5	2	2	11	8
Intermediate risk	109	42	58	45	51	39
Poor risk	54	21	26	20	28	22
Risk not known/not done	73	28	38	29	35	27
Missing	10	4	5	4	5	4
Bone Marrow Morphology - % Blasts						
Mean	46.2		48		44.4	
SD	28.4		27.7		29.1	
Hemoglobin, g/L						
Mean	131.1		120.9		141.1	
SD	184.9		167.5		200.9	
Platelets, 10^9/L						
Mean	85.4		78.1		92.7	
SD	131.2		79.2		167.7	
WCC, 10^9/L						
Mean	14.1		15.6		12.6	
SD	24.6		29		19.4	
Neutrophils, 10^9/L						
Mean	3.1		3		3.2	
SD	9.2		8.4		9.9	

Table 2. Univariate and multivariate analysis of predictors of CR and OS in the study population

Overall Response					
	Covariate	Univariate analysis		Multivariable analysis*	
		OR (95% CI)	*P* †	OR (95% CI)	*P*
Clinical variables	*Disease Status*		(<0.001)		
	Refractory (v Relapsed)	0.2 (0.1, 0.9)	0.03	Not estimable	
	Newly diagnosed (v Relapsed)	2.1 (1.0, 4.6)	0.051	3.6 (1.1, 11.7)	0.037
	Baseline WBC				
	≥10 (v <10)	0.7 (0.3, 1.5)	0.39	0.5 (0.2, 1.8)	0.292
	Cytogenetic Risk		-0.416		
	Intermediate (v Poor)	0.6 (0.3, 1.3)	0.204	0.6 (0.2, 2.0)	0.424
	Favorable (v Poor)	1.0 (0.2, 4.9)	0.951	0.8 (0.1, 5.6)	0.843
	Age				
	≥70 (v <70)	1.3 (0.7, 2.5)	0.447	1.3 (0.4, 3.8)	0.674
	Diagnosis				
	AML (v MDS)	0.8 (0.4, 1.8)	0.649	1.0 (0.3, 3.7)	0.957
	ECOG P.S.		-0.98		
	1 (v 0)	1.0 (0.5, 1.9)	0.92	1.6 (0.6, 4.4)	0.395
	2 (v 0)	1.1 (0.4, 3.2)	0.902	1.0 (0.2, 5.8)	0.981
Mutations	*STAG2 mutation*				
	Present (v Absent)	0.2 (0.1, 0.6)	0.002	0.3 (0.1, 1.4)	0.117
	IDH2 mutation				
	Present (v Absent)	0.4 (0.2, 0.9)	0.029	0.4 (0.1, 1.3)	0.139
	NPM1 mutation				
	Present (v Absent)	2.5 (1.0, 6.2)	0.038	8.6 (1.6, 45.8)	0.012

Overall Survival						
	Covariate	Median OS	Univariate analysis		Multivariable analysis**	
		(95% CI) , months	HR (95% CI)	*P* ‡	HR (95% CI)	*P*
Clinical variables	*Diagnosis*					
	MDS	19.4 (11.3, 22.7)	1		1	
	AML	9.1 (8.0, 11.1)	2.0 (1.3, 3.0)	0.0008	2.3 (1.3, 4.3)	0.007
	Baseline WBC					
	<10	11.5 (9.8, 13.6)	1		1	
	≥10	8.8 (6.7, 10.5)	1.5 (1.1, 2.0)	0.0116	2.2 (1.4, 3.5)	0.001
	Disease Status			-0.0132		
	Relapsed	7.6 (6.4, 10.5)	1		1	
	Refractory	9.8 (8.3, 13.2)	0.8 (0.5, 1.2)	0.218	1.0 (0.5, 1.8)	0.976
	Newly diagnosed	11.7 (10.1, 14.9)	0.6 (0.5, 0.9)	0.005	0.5 (0.3, 0.8)	0.003
	ECOG P.S.			-0.0235		
	0	12.7 (9.6, 19.4)	1		1	
	1	10.1 (8.0, 11.5)	1.6 (1.1, 2.1)	0.009	1.6 (1.0, 2.6)	0.035
	2	9.5 (7.8, 15.4)	1.5 (0.9, 2.4)	0.0968	1.6 (0.9, 2.9)	0.131
	Age					
	<70	9.3 (7.6, 11.6)	1		1	
	≥70	11.1 (9.0, 13.5)	0.8 (0.6, 1.1)	0.1706	1.6 (0.9, 1.8)	0.448
	Cytogenetic Risk			-0.8589		
	Poor	9.5 (7.1, 11.1)	1		1	

	Intermediate	11.4 (8.1, 15.3)	0.9 (0.6, 1.3)	0.6367	1.2 (0.7, 1.9)	0.549
	Favorable	12.0 (1.7, N/E)	0.8 (0.4, 1.8)	0.6392	1.1 (0.5, 2.8)	0.802
Mutations	*CDKN2A mutation*					
	Absent	11.0 (9.3, 12.6)	1		1	
	Present	4.5 (0.2, 7.8)	3.9 (1.9, 8.0)	0.0001	10.0 (3.3, 30.3)	<0.001
	TP53 mutation					
	Absent	11.3 (9.4, 13.0)	1		1	
	Present	7.6 (2.4, 9.6)	1.8 (1.2, 2.6)	0.003	4.7 (2.5, 9.0)	<0.001
	IDH1 mutation					
	Absent	11.1 (9.4, 12.7)	1		1	
	Present	5.6 (2.8, 9.8)	1.9 (1.2, 2.9)	0.004	3.6 (1.7, 7.6)	0.001
	NPM1 mutation					
	Absent	11.1 (9.1, 12.6)	1		1	
	Present	8.1 (5.6, 10.7)	1.5 (1.0, 2.2)	0.037	0.6 (0.4, 1.1)	0.122
	FLT3ITD mutation					
	Absent	11.1 (9.0, 12.7)	1		1	
	Present	8.8 (6.1, 11.6)	1.5 (1.0, 2.1)	0.04	1.0 (0.6, 1.8)	1

OR, Overall Response; WBC, White Blood Cell, ECOG P.S, Eastern Cooperative Oncology Group Performance Status, N/E, Not estimable

* Logistic regression model adjusted for all variables in the table

† Given by the Chi-square or Fisher's exact test, corresponding to pairwise comparisons or the overall comparison as indicated between parentheses

** Cox Proportional Hazards model adjusted for all variables in the table

‡ Given by the Log-rank test, corresponding to pairwise comparisons, or the overall comparison as indicated between parentheses

Figure Legends

Figure 1

Consort Diagram

Schematic representation of patient disposition in the trial

Figure 2

Overall Survival of Trial Patients

(a) Survival in all study patients

(b) Survival in patients with AML

(c) Survival in patients with MDS

Figure 3

Mutation profile of study population and correlation with clinical response.

(a) Frequency of mutations (as % of patients) in patients at trial entry. Patients are further divided according to best response achieved.

(b) Frequency of number of mutations detected per patient pre-treatment.

(c) Comparison of mutations detected in RAvVA cohort compared with recently published AML cohorts (Papa: Papaemmanuil et al(19), TCGA(20), Welch et al(21)).

(d) Correlation of combinations of detected mutations with CR and OS: only genes where there were at least 5 patients with 2 mutations are included in this analysis. Unadjusted *p* values from a Fisher exact test are shown. The top right half of the table (values in shades of green) show mutation combinations significantly

associated with decreased OS. The bottom left half of the table (with values in shades of yellow) shows absence of significant mutation combinations predictive of CR. Key: CR (includes CR, CRi), PR, NR (no response including stable disease and progressive disease), ID (induction death), NA (response data not available).

Figure 4

Flow cytometric measurement of LSC populations.

(a) Quantitation of expanded CD34+ progenitor or CD34- precursor LSC populations in AML patients pre-treatment.

(b) Quantitation of LMPP-like LSC pre-treatment and at CR expressed as fold change of upper limit of LMPP frequency in normal bone marrow (upper limit of normal, ULN, dotted line, assessed in 12 normal donors).

(c) Longitudinal quantitation of LMPP-like LSC in patients pre-treatment, at CR (multiple time points in 2 patients: CR’ and CR’’) and at relapse.